

REMARKS/ARGUMENTS

In response to the Office Action of August 30, 2005, Applicants have amended the claims, which, when considered with the following remarks, is deemed to place the present application in condition for allowance. Favorable consideration of all pending claims is respectfully requested.

The Examiner has acknowledged Applicants' election of claims in the response to the restriction requirement filed on May 10, 2005. Therefore, group II, claims 14-16 and 18 have been withdrawn from consideration by the Examiner. As presently amended, claim 14 recites a process for purifying on a large scale a product from a feedstock wherein the product is a rapamycin or a derivative thereof or an ascomycin or derivative thereof. Cyclosporin, which is not part of the elected subject matter, is no longer recited by this claim. Applicants therefore respectfully request that the Examiner reinstate claim 14. In addition, since claims 15 and 16 depend from presently amended claim 14, Applicants respectfully request that the Examiner also reinstate these two claims.

In the Office Action, the Examiner has stated that the Information Disclosure Statement (IDS) filed on 9/16/03 fails to comply with 37 CFR 1.98 (a)(1). The Examiner has therefore placed the IDS in the application but information referred to therein has not been considered. It is respectfully submitted that the IDS previously submitted contained all of the necessary information to identify the references cited by the Applicants so as to allow the Examiner to consider the art. Moreover, as of the submission date of the IDS, **September 16, 2003**, there was no requirement under 37 C.F.R. 1.98(a)(1) for "a column that provides a blank space next to each document to be considered, for the examiner's initials. *See* 37 C.F.R. §1.98 "[...]" paras. (a) and (c) revised and par. (e) removed, 69

FR56481, Sept. 21, 2004, **effective Oct. 21, 2004.**] Thus, as of September 16, 2003, the submission date of the IDS in this application, the IDS was in compliance with 37 C.F.R. §1.98 in effect at the time.

Even if there was a requirement in place at the time for a blank space next to each document to be considered for the examiner's initials, the IDS contained ample space for the Examiner to initial each reference after the art was considered. Nonetheless, in order to expedite prosecution of the present application, a newly prepared IDS containing the same information as previously filed has been submitted herewith. It is not believed that any additional fee is required, but if an additional fee is required, please charge the same to Deposit Acct No. 04-1121.

The Examiner has objected to Claims 21 and 22 as allegedly substantially duplicative of claims 19 and 20. Claims 19-22 are presently canceled from the application. The objection to claims 21 and 22 is therefore moot.

Claims 11-13, 19 and 21 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter of the invention. In particular, the position of the Examiner is that the phrase "a rapamycin or an ascomycin" is indefinite because use of the articles "a" and "an" make it appear that Applicants are intending to claim two genera of compounds. According to the Examiner however, it is unclear what members belong to the two genera. The Examiner has inquired how the genera differ from the compounds (i.e., a rapamycin and an ascomycin). For example, the Examiner has stated on page 4, paragraph 8 of the Office Action that the fourth paragraph on page 3 of Applicants' specification "merely lists these five compounds but does not describe the families." The

Examiner has also inquired how FK506 can be an ascomycin, noting that FK506 has a trans double bond in the macrolactone skeleton while ascomycin has a cis double bond at the same position.

As presently amended, claim 11 recites “or a derivative thereof” after each of the terms “rapamycin” and “ascomycin”. The articles “a” and “an” as they appeared before “rapamycin” and “ascomycin” have been canceled from the claim. Support for these amendments may be found throughout the specification, e.g., page 3, lines 18-24. In addition, Applicants submit herewith as Exhibit A, Uchida, T. et al. (2002) “Identification of genes coding enzymes for ascomycin tetra-hydropyranose ring formation” *Internat. J. Mol. Med.* 9:141-145” that describes the relationship of the compounds recited in the presently amended claims. As described on page 141 of the paper, Ascomycin is a C-21 ethyl analog of FK506 and is a 23-member macrolide. Ascomycin is also known as immunomycin and FK520. *See* Figure 1. As may be seen from the dates of the references cited on page 141 of the paper, the relationship among ascomycin and derivatives thereof, and rapamycin and derivatives thereof, was known in the art at the time the present application was first filed. In view of the amendments to the claims and the foregoing remarks, withdrawal of the rejection of claims 11-13, 19, and 21 under 35 U.S.C. § 112, second paragraph is respectfully requested.

Claims 17, 20 and 22 have been rejected under 35 U.S.C. § 112, second paragraph, because the limitation “FK506” is recited in the last line of each of these claims. It is the Examiner’s position that no antecedent basis exists for this term. As stated above, Claim 11 has been amended to include the phrase “or a derivative therefore” after each of the terms “rapamycin” and “ascomycin.” Since FK506 is a

derivative of ascomycin (*see* specification, page 3, lines 23-24), claim 11 as presently amended provides the necessary antecedent basis for this term with respect to claim 17. Claims 20 and 22 have been canceled from the application, and the rejection of these claims is therefore moot. In view of the foregoing, it is respectfully requested that the rejection of Claim 17 under 35 U.S.C. § 112, second paragraph, be reconsidered and withdrawn.

Claims 21 and 22 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly indefinite for ultimately depending from non-elected claim 14. Claims 21 and 22 are presently canceled from the application. The rejection is therefore moot.

Claims 11, 19 and 21 have been rejected under 35 U.S.C. § 102 (b) as allegedly anticipated by U.S. Patent 5,359,060 to Hauske.

Hauske has been cited for teaching purification of FK520 by counter current distribution using 10:1 heptane:acetonitrile solvent system. In making the rejection, the Examiner has directed Applicants' attention to column 9, lines 54-59 and column 10, lines 40-43.

Column 9, lines 54-62, provides:

The concentrate was subjected to four tube counter current distribution in 20 liter carbuoys using 10 liter top layer and 1 litter bottom layer per carbuoy of a heptane/acetonitrile 10/1 system. The active bottom layers were collected, combined and concentrated. The material was further purified via filtration through Florisil (washing with hexane, hexane/methylene chloride and methylene chloride, successively, with a gradual increase in methylene chloride).

It is noted that in making the rejection, the Examiner included Claim 11 as part of the set of claims being rejected, yet concluded that only Claims 19 and 21 were taught.

For the sake of completeness, this response assumes that the Examiner's conclusion reached in this rejection also pertains to Claim 11.

In contrast to Hauske, claim 11 of the present application recites that heptane and acetone or heptane and isopropanol is used in the lighter phase of the counter current separation and water and acetone or water and isopropanol is used for the heavier phase. Nowhere in Hauske is there a teaching for the use of two different solvent mixtures for the two different phases, wherein the lighter phase flows counter to the heavier phase. Moreover, nowhere in the reference is the mixture of these solvents even mentioned. Claim 11 is therefore distinguished from the teaching provided by Hauske. Withdrawal of the rejection of claim 11 under 35 U.S.C. § 102 (b) is therefore warranted. Claim 19 and 21 have been canceled from the application and the rejection as pertains to these two claims is therefore moot.

Claims 19-22 have been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by EP 652,219 A1 (hereinafter "Gletsos"). Gletsos teaches the purification of both Rapamycin and FK506 by extraction. In contrast, claims 19-22 recite rapamycins and ascomycins produced by counter current separation. Citing the MPEP 2113 and *In re Thorpe*, 777 F.2d 695, 698, 227 USPQ 964,966 (Fed. Cir. 1985), the Examiner has asserted the proposition that patentability of a product does not depend on its method of production, but rather non-obvious differences in the product. Applicants respectfully submit that claims 19 to 22 are directed to products that are novel and non-obvious over Gletsos. In order to advance prosecution of this application however, and not in any way acquiescing to the position of the Examiner, claims 19-22 have been cancelled without

prejudice. Applicants reserve the right to file one or more divisional or continuation applications directed to the subject matter of the canceled claims.

Claims 19-22 have been rejected under 35 U.S.C. § 102 (e) as allegedly anticipated by U.S. Patent 5,616,595 to Chu et al. Since claims 19-22 have been canceled from the application, the rejection is now moot.

Claims 19-22 have also been rejected under 35 U.S.C. § 102 (b) as allegedly anticipated by EP 427680 A1 to Baumann. The rejection of claims 19-22 is moot since these claims have been canceled from the application.

Claims 19-22 have been rejected under 35 U.S.C. § 102 (e) as allegedly anticipated by U.S. Patent No. 5,665,772 to Cottens et al. As these claims have been cancelled from the application, the rejection is moot.

Claims 19 and 21 have been rejected under the judicially created doctrine of obviousness-type double patenting as allegedly unpatentable over claim 1 of U.S. Patent No. 6, 706,727. The rejection is now moot since claims 19 and 21 have been cancelled from the application.

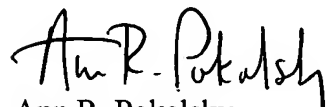
Applicants acknowledge the Examiner's finding that Claims 12, 13 and 17 contain allowable subject matter and would be allowed if rewritten to overcome the rejection(s) under 35 U.S.C. § 112, 2nd paragraph.

Accordingly, in view of the foregoing remarks and amendments, the present

application is believed to be in condition for allowance, which action is earnestly solicited.

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Identification of genes coding enzymes for ascomycin tetra-hydropyranose ring formation

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Abstract. Macrocyclic polyketides have generated great interest in biosynthetic chemistry because of the structural complexity and medicinal activities. The synthetic genes consist of the number and type of active sites of modular polyketide synthases. The cosmid library - prepared with the ascomycin (an antibiotics with immunosuppressive activity) - producer, *Streptomyces* sp. AA6554 genome was screened with an ascomycin ketosynthase gene probe, and one and a half modules were isolated. Database analysis shows that one of the modules consists of the genes coding a series of enzymes for the tetra-hydropyranose ring synthesis.

Introduction

Polyketides are natural products identified in various species and are especially abundant in fungi and actinomycetes. Genetic analysis of polyketide genes separated them into two classes (1). One of the class consists of macrolides which provide an excellent source to the pharmaceutical drugs. Ascomycin, C-21 ethyl analog of FK506 (2) is a 23-member macrolide (3). Ascomycin is also known as immunomycin and FK520. FK506 and rapamycin consisting of similar structures to that of ascomycin (Fig. 1) have potent immunosuppressive properties to inhibit T cell activation both *in vivo* and *in vitro* (3,4). These three compounds contain the pyranose-pipecolinyl region (C1 to C15; Fig. 1) which mimics leucine- (twisted amide) -proline peptide where peptidyl prolyl cis/trans isomerase (PPIase) binds to and causes various biological activities (5).

We identified the genes coding the synthetic enzymes for ascomycin tetra-hydroxypyranose ring, a part of pyranose-

pipecolinyl region where it binds to FK506-binding protein. The structure of the module of this gene is different from those of the FK506 synthase gene A, *fkBA* (6) and rapamycin synthase gene 3, *RAPS3* (7,8) which code the synthases for tetra-hydroxypyranose ring for FK506 or rapamycin respectively.

It is important to increase the genetic database of macrocyclic polyketide synthases. Such information will make it possible to manipulate the synthase genes, generate unnatural macrolides and increase the diversity of macrolides dramatically.

Materials and methods

Cloning. Genome DNA was isolated from *Streptomyces* sp. AA6554 and digested with *Sau3A* partially. The digested DNA was ligated into pWE15 cosmid with *Bam*HI sites at the ends (Stratagene). Ascomycin synthetic gene cluster was isolated by using the ketosynthase (KS) gene as a probe. The ascomycin KS gene was cloned by PCR. The primers for the PCR (5' primer, 5'-TTCGGGATCAGTCCTCG-3'; 3' primer, 5'-AGGATGACGTGGGCGTT-3') were designed to cover the highly conserved region of the KS gene by comparing the sequence of the KS gene for DEBS1 (2) and that for RAPS3 (7). The amplified product (1047 bp) was sequenced, compared with the other KS genes and confirmed to be a KS gene. The cosmid library was screened with the ascomycin KS gene as the probe. One of the positive clones was picked up, fragmented with sonication and subcloned into pUC19 plasmid to be sequenced.

Sequence analysis. The DNA sequencing was done on double-strand DNA templates with dideoxy method using an automatic sequencer (Applied Biosystems, Model 377 sequencer). The random sequences were compiled and the assembly was performed with the Applied Biosystem Auto Assembler (ABI). The deduced protein sequences were compared with sequences in the GenBank database using the BLAST program (9) and the alignments were performed using the PILEUP and CLASTW program (10).

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Key words: polyketide, ascomycin, tetra-hydropyranose ring

Results and Discussion

Streptomyces sp. AA6554, high producer of ascomycin was newly isolated from soil. We speculated that the biosynthetic

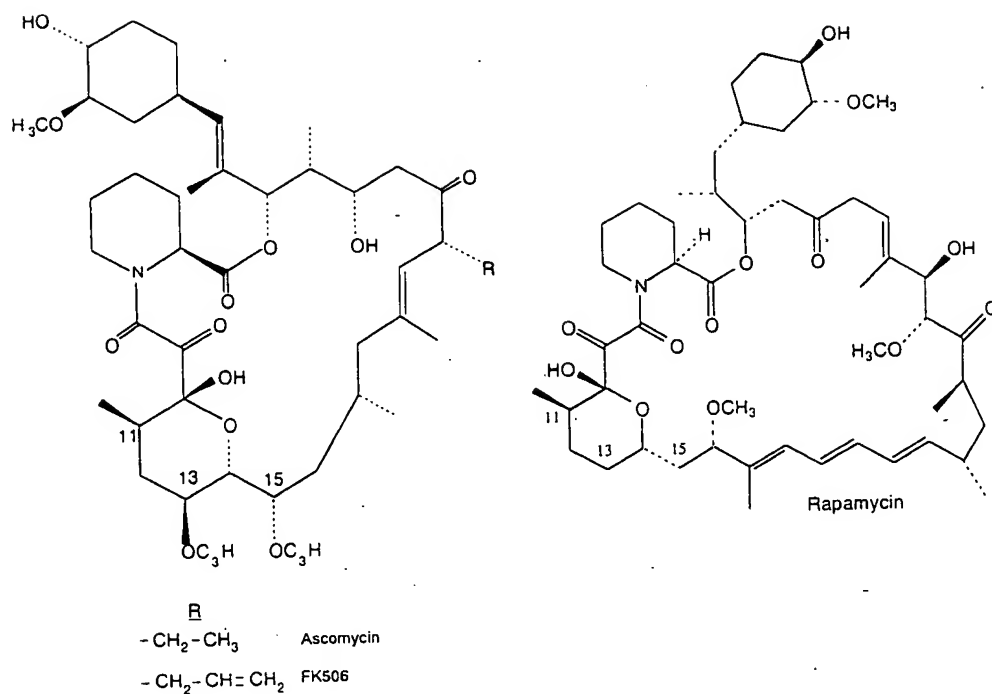


Figure 1. Structure of ascomycin, FK506 and rapamycin. Ascomycin, C-21 ethyl analog of FK506 is a 23-member macrocyclic lactone. Ascomycin is also known as immunomycin and FK520.

	ASC	FK506	RAPS	Consensus
ASC	REAGRLSEAS GRGSAHNSA LRRHDSQB PVAVVGNACR YPGGASPSA LNRLLASGAE ANAKPTDRG WDLGLFPHD PDRPTSHAR EGGFLYDAD PDPPTGISP ASALVCPQD	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR
FK506	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR
RAPS	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR
Consensus	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR	...GTRAPVA AR...TAA TAAKD...B PLAIYGNACR LPGGVASPQS LNRLLASGTD AITRPPADRQ WDVGLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR EGGFLYDAD PDAIGTFVR

ASC	ATTPFAAEP	TTAWPPAGAV	PVDVAOLIER	LTAQGTATCP	AFEGLEBTANR	LGEBNFAEVR	LAFEREGEAD	ATGVNPFALLO	SALBPVBELE	SGGVRVDPGE	ATVLEPSPG	GVRLEPTGAT
fkA	RVFQF...AV	STAWPPAGAV	PA...D...	...GLPQANR	AAQGVVFAE	VOSP...D	GFVANFDLLO	AVFS...A...	...VGDG...S	...RQPTGHR	DLAVASDADT	
RAPS	GTVSG...VQA	GSANPPGAV	PVETGVF...	...SLQVVR	RGHEVFAEVA	LOST...HDAT	TYALRFALLT	AALT...	...TAG...	...ETPAANR	ALTLNHRPA	
Consensus	-----	---ANPP-GAV	P-----	---L---VR	-----	-----	---RF-L-	-----	-----G-	-----	-----	
ASC	RLRVITITPA	PDVITLITD	ONGAPVATD	SLGLREVPAD	ANRVSNSATA	DAPLTELONG	PYPVSASAV	FTANRVLVGA	RBPDLGLPAN	FDLAALRAAL	DDGEPVPOVV	VLACPGATD
fkA	VLACITARD	SGVILAAFD	GAGMPVLTA	SVTLGEVASA	GG...SDRS	DG...LRLAHL	P...VAREAT	DGADSELECY	T...LITATH	P...DD...	...FDD	
RAPS	SLRVLISSD	DGTLSDVATD	SYGLPVLTVR	SLTLRTVPVT	SP...ATS	TDLLTLTMA	ETAPQSTGL	TVGRFEDLVS	D...ADVPVFEV	AVFT...	...ALPOS	
Consensus	---LF---	---D---	---PV---	S---L---V---	-----	---L-L-W-	-----	-----	-----	-----	---F---	
ASC	SADPDQTPAN	VRAAEVPLG	ALRTNLTQDR	PSGARLVAT	RGAVATGPGD	APADLATAPV	NGLVRAAQA	RFQRVHLLDL	DDCTASRDAL	RTAFPAANAD	ABSELAVRAG	TABVPLVNR
fkA	PTNPHTPTT	THQPTTEVLT	ALQHLITTM	RT...LIVHT	...TTQPPG	...AAV	TGLTRTAQNE	RFQRVLIET	HEHTF...	...LPLTQLTL	NQPLRLTMM	TLTPBLTPI
RAPS	SENF...LEQ	P...VAVDPOV	AVQTLGGER	PTOSTLVVPT	G...TLAAAG	...V	SLKRSASQE	RFQRVPLVES	DDTLAP...	...DLAATVGL	DFPLRVSGD	RTPAPLHAP
Consensus	-----	---VL-	A-----	---L-T	-----	-----V	---GL---AQ-	RF-R-	-----	---L---	-----	---L---
ER												
ASC	RPDRD...T	P...ARALDPDG	TALITGGTGA	LORLVARNLV	TANGVRELL	VSRFGGITO	IDAFADBLTS	LGAADVRYTA	CDAAOPHALA	VLLATLPEAR	PLTAVVHAAG	VDDGVVTSN
fkA	TYHNTITTT	PHPTPLNPH	AILITGGSGT	LAQILARHNL	SPH...TTL	LSRTF...PF	...FIT	PGTHIP...	CDLDTPTQIT	QALTEIPQ...	PLTGTETAA	TIDDTATML
RAPS	NASGS...E	P...EAVMDPOV	TVLITGGSOV	LAGIAARHNV	ARGVRRHLL	LSRSLAPAL	IN...QLG	LGARVET...AA	CDVSDRAALA	QVLAGVSPR	PLTAVHETAG	VDDGVVTSN
Consensus	-----	P-----	---LITGG-G-	L---ARHL-	---L	---SR-	-----	-----	---CD--D-	---L---	---PLT---R-A-	---LDD-----
ASC	TPQQLDTVLA	FELDAAPHE	RLTRTKDPA	PVNFSSAAAT	NGMGQAMTA	AANMFLWALA	SERRAIGRAS	HALAVGLIAS	AGCMTGELSD	ADLARNHARS	IAPVSHIQA	TLLDTALTO
fkA	TPQQLDTVLA	PAADAAPHE	HTQQLPLTH	PVLTSSAAAT	LGSPQAMTA	AANAPDLALA	TTJANGHMT	TYLTSQSLTD	SRRDPRRGG	PLPSDDHGN	TLDAVAGSG	
RAPS	TAQQLDTVLA	PAADGAHE	ELTRNTDLAA	PVNTSSAAAG	NGMGQAMTA	AANAPDLALA	SERRAIGLAS	IRGGGLGLED	TGSLTQTLQ	LDGSLTAVR	LTATATAGN	RLPDATVRS
Consensus	T---L-T-L-	PE-D-AHEH	---T---	EV--SSAA--	-G-CQ-NYA	AA--PL-ALA	-----G-	-----G-	---T-L-D	---D---	---R-G	---D---
ACP												
ASC	HALVLPARFD	LAALTRAAT	GFLPFLREL	VHVPTRAPY	TGANSLSBL	AGLPAGEGR	LVHSLVRDQV	ATVLAHFAPE	AIBPKAFQD	LCPFSLTALD	LNNRLAATG	IRIPATVIFD
fkA	EDVLAAMKD	PAQ...NA	GDVPLISLG	RKSARRTAT	G...QTAPQL	ALPAAEDKT	ALVTLVEDAT	AAVLGHADAS	GIAPPTTFKD	LGLDSTLVA	LNNRLAATG	IRIPATVIFD
RAPS	NPILVAAPFD	PVMD...A	EVPALLRSL	RPVARRAST	S...OSSARVL	AAAPARBD	ALLVLRDSDA	ALVGLHADAS	TIPAAAPFD	LGLDSTLVA	LNNRLAATG	IRIPATVIFD
Consensus	---A-D	-----	---R-T	---R-T	---R-T	---R-T	---LV-D-	A-VL-H-	-----	---LGLDSTLVA	LNNRLAATG	IRIPATVIFD
KS												
ASC	TFPQALVGT	LRRLTGAPA	AAPLFTATA	AAADDPIVI	VGNACRYPG	AGSPALFRL	VADGVDAIGE	PPGDRGMDLA	GLFDFPDHDT	GTSTARBGOT	LTSAPFZAS	FFUGISPRAL
fkA	ETPFAVLAAS	LRTDLEGT	AAAPL...ATA	RTNDEPLAI	VGNACRYPG	VSPFDFLRL	VASGTDALTE	PPDRGMDID	RKFPDOPDAP	GETTVRHGG	LTSAPFZAS	FFUGISPRAL
RAPS	TFPFAVLAAS	LRTDLEGT	AAAPL...ATA	RTNDEPLAI	VGNACRYPG	VSPFDFLRL	VASGTDALTE	PPDRGMDID	RKFPDOPDAP	GETTVRHGG	LTSAPFZAS	FFUGISPRAL
Consensus	---TF---L---	L---G---	---AP---	---D---	VGNACRYPG	---SPF-LRL	V--G-DAL-	PP-DRGMD-	---PDOPD-	G---T---GGP	L---A---FDS	FFUGISPRAL
ASC	ATDPQQLLL	ETAWAPESA	GIDPVSLRG	SAVITGVNY	DYQSRFLGR	TREGVGRHL	TGSTFISASG	RYAFTVGLRG	PAVTVDTACS	SSLVAMHAA	QALRQECTL	ALAGGVTVNA
fkA	ANDPQQLVIL	ETVWAPESA	GIDPVSLRG	DTGVTHGAP	HTG...G	AGDGLGGTA	TATQNSVLGS	ELSTFFGHRG	PAVTVDTACS	SSLVAMHAA	QALRQECTL	ALAGGVTVNA
RAPS	ANDPQQLVIL	ETVWAPESA	GIDPVSLRG	DTGVTHGAP	HTG...G	AGDGLGGTA	TATQNSVLGS	ELSTFFGHRG	PAVTVDTACS	SSLVAMHAA	QALRQECTL	ALAGGVTVNA
Consensus	A-DPQQL--	E-W--FF-A	G-I-P--RG-	---G---	---TG---	---G---	T---SSG	R---FG-EG	PA-T-DTACS	SS-VA-R-A-	---LR-QEC-L	AL-GGVTVNA
ASC	TFNTVPSR	QRLGAPDGR	KFFAAADGT	QWGGIGLV	LSRLSDARRN	GRVLAIVRG	SAVMDQASN	GLTAPUGPSQ	QRVIRQALAN	AKLSPABVDA	VSABGTGTL	GDPIBAQAL
fkA	TFNTVPSR	QRLGAPDGR	KFFAAADGT	QWGGIGLV	LSRLSDARRN	GRVLAIVRG	SAVMDQASN	GLTAPUGPSQ	QRVIRQALAN	AKLSPABVDA	VSABGTGTL	GDPIBAQAL
RAPS	TFNTVPSR	QRLGAPDGR	KFFAAADGT	QWGGIGLV	LSRLSDARRN	GRVLAIVRG	SAVMDQASN	GLTAPUGPSQ	QRVIRQALAN	AKLSPABVDA	VSABGTGTL	GDPIBAQAL
Consensus	TF---VEP-R	QRLGAPDGR	KAF---ADGT	---BG-G-L-	LSRLSDARRN	G---VLA--R-	SAVMDQASN	G---AFNPGSQ	QRVIRQALAN	A-L---AL-	A-L---VD-	VSABGTGTL
ASC	ATTGRERPED	RPLMDSIRS	NICRTQAAAG	VAGVIRKVA	HREGLLPXL	HIDRFSQEV	NODGQVILLT	BAVENFRABR	PRRAVSSYG	ISGTANVIL	EQAPORAFDT	GRKPFEDDP
fkA	ATTGQDR...D	TYLIGSVAS	NICRTQAAAG	VAGVIRKVA	HREGLLPXL	HIDRFSQEV	NODGQVILLT	BAVENFRABR	PRRAVSSYG	ISGTANVIL	EQAPORAFDT	GRKPFEDDP
RAPS	ATTGQDR...E	TYLIGSVAS	NICRTQAAAG	VAGVIRKVA	HREGLLPXL	HIDRFSQEV	NODGQVILLT	BAVENFRABR	PRRAVSSYG	ISGTANVIL	EQAPORAFDT	GRKPFEDDP
Consensus	ATTG--R---	---L-LGS--S	NIGH-Q--G	---GVIRKVA	---R--P-L-	R-DPS-HV-	R--G-V-L-	S---NF--R	PRRA-VSS-G	ISGTANVIL	E-----	-----
AT												
ASC	E...VVPVLS	ARGATALRD	APALVARIAT	GPLASSABG	YSLIKSRFL	DERAVVGGD	EALTAALBA	LAAGSRPVC	VGPQAVVSGD	GVPQVLPVFG	QCSQVWVGA	GLDASPVV
fkA	...LVPLPS	ARTSSALQ	YHRLGVRG	...ARLAAVA	DGLVGRVTF	CHRAVLGDS	...TVAG...	VAGABSR...	...T...	...VEVFP	QCSQVWVGA	GLDASPVV
RAPS	ASDVPLVLS	ARTSSALQ	YHRLGVRG	...ARLAAVA	DGLVGRVTF	CHRAVLGDS	...TVAG...	VAGABSR...	...T...	...VEVFP	QCSQVWVGA	GLDASPVV
Consensus	---P-S-A	---L---	---	---	---	---	---	---	---	---V-VFP	QCSQVWVGA	GLDASPVV
ASC	ARVACBAPAL	APVGVWJTD	VLRGVDAAG	LGRVGVVQV	LWAVWVSLA	VNARCVYRA	AVVGVSGGI	AAACVACALT	LEDGARVVAL	RSAPLR...LA	GGGANASIAL	GCRRVGLLS
fkA	ARVACBAPAL	APVGVWJTD	VLRGVDAAG	LGRVGVVQV	LWAVWVSLA	VNARCVYRA	AVVGVSGGI	AAACVACALT	LEDGARVVAL	RSAPLR...LA	GGGANASIAL	GCRRVGLLS
RAPS	ARVACBAPAL	APVGVWJTD	VLRGVDAAG	LGRVGVVQV	LWAVWVSLA	VNARCVYRA	AVVGVSGGI	AAACVACALT	LEDGARVVAL	RSAPLR...LA	GGGANASIAL	GCRRVGLLS
Consensus	-R-EC---L	---R-L-	VL---	---V-V-QP-	---LA-VSLA	---GV-F-	AV-GSGGI	AAACVACA-	L-D-AR-V-L	RS-...LA	G-GANASIAL	---VE---
ASC	GLGDRVAAY	VAAVGPAST	VYSGPFIEQA	AAVAAACRGT	RRARKHVDY	ASRSPQVBI	AGELCBVIRG	VEPVGVSGG	VVFSTVSGG	RVDAVLSGG	TVNHLNRY	RFAATIGLL
fkA	G...V...VM	VAAVGPAST	VYSGPFIEQA	AAVAAACRGT	RRARKHVDY	ASRSPQVBI	AGELCBVIRG	VEPVGVSGG	VVFSTVSGG	RVDAVLSGG	TVNHLNRY	RFAATIGLL
RAPS	G...V...VM	VAAVGPAST	VYSGPFIEQA	AAVAAACRGT	RRARKHVDY	ASRSPQVBI	AGELCBVIRG	VEPVGVSGG	VVFSTVSGG	RVDAVLSGG	TVNHLNRY	RFAATIGLL
Consensus	G-----	---AL-NGP-ST	V--G-F--V-	-----	---R-L-VDT	ASH---V-I	---L---	---V---	---TV-G-	---V---	TV-NHLNRY	---A---L-
ASC	GAGRVVIFV	STHVLTHGH	QITTEAGVT	ALTYPIERD	HGDIQVLT	LAQAFCTAGD	LWTTLPHTD	PAPRTVPLT	TAPOQRHML	SGSAGRGDP	TDGLGVGSD	PLGAAVSLA
fkA	GS...LPIBC	SABVPLPAL	DQ.....	...EAVV	ABRVVLQPA	SWAVVSLA	VLAQBGVSPD	AVVGVSGGI	AAACVACALT	LEDGARVVAL	RSAPLR...LA	GGGANASIAL
RAPS	GS...LPIBC	SABVPLPAL	DQ.....	...EAVV	ABRVVLQPA	SWAVVSLA	VLAQBGVSPD	AVVGVSGGI	AAACVACALT	LEDGARVVAL	RSAPLR...LA	GGGANASIAL
Consensus	---G-S---	---PVL---	---	---TV---ER-D	---G---	---	---	---	---	---	---	---
ASC	DGSEILITGH	LTAGGAGVL	COHVAGTFL	VPGAQVWLA	LRAADBAGG	TIBELALQVP	LVLPTDGGV	VQVVGVAAD	DGRDVRVVS	RPD...HDA	---	---
fkA	THGCTVLTOR	ISLA...THFL	ADHVRGVL	LPGTATVRL	VRAADVEIC	VQVBLVSTP	LLLPQTGGVQ	LSVSVARADE	SGHRTVTVPS	QANDTDVIA	VVSATISTSD	TLPLSFESD
RAPS	THGCTVLTOR	ISLA...THFL	ADHVRGVL	LPGTATVRL	VRAADVEIC	VQVBLVSTP	LLLPQTGGVQ	LSVSVARADE	SGHRTVTVPS	QANDTDVIA	VVSATISTSD	TLPLSFESD
Consensus	---RA-DE--C-	---BL---	---P-P-TQ-V	---V-V---	---G-R-V-V---	---	---	---	---R-V-V---	---	---	---
ASC	QVPPCTPAL	QVTEPAQIA	ALGRTVPTT	RGILAAARAG	DTYVAVPAL	EDRAADAPH	GVRPALLDAA	LQSGSLNLE	SDGSGVQLP	FSNHRVPHH	TGATSLVIA	VPGDGGRLR
fkA	QVPPCTPAL	QVTEPAQIA	ALGRTVPTT	RGILAAARAG	DTYVAVPAL	EDRAADAPH	GVRPALLDAA	LQSGSLNLE	SDGSGVQLP	FSNHRVPHH	TGATSLVIA	VPGDGGRLR
RAPS	QVPPCTPAL	QVTEPAQIA	ALGRTVPTT	RGILAAARAG	DTYVAVPAL	EDRAADAPH	GVRPALLDAA	LQSGSLNLE	SDGSGVQLP	FSNHRVPHH	TGATSLVIA	VPGDGGRLR
Consensus	TVPPAQAPM	HYADTDRLH	AGRTVOPAT	QGLQAARAG	DTYVAVPAL	EDRAADAPH	GVRPALLDAA	LQSGSLNLE	SDGSGVQLP	FSNHRVPHH	TGATSLVIA	VPGDGGRLR

Figure 2. Comparison of the deduced amino acid sequence of ascomycin synthase gene with those of *fkA* and *RAPS3*. The consensus sequence is shown under their sequences. One complete module containing a KS, an acyltransferase (AT), a dehydratase (DH), an enoyl reductase (ER) and an acyl carrier protein (ACP), and a part of the module containing a KS and an AT were identified.

genes for ascomycin consist of a complex of modules like other macrolides, such as erythromycin (2,6), rapamycin (7,8) and FK506 (11). The sequence of β -ketoacyl synthase

(KS) gene of ascomycin synthetic genes is similar to those of the other macrolide synthetic genes. The amino acid sequence of KS of 6-deoxyerythronolide B synthase (DEBS) (2) and that

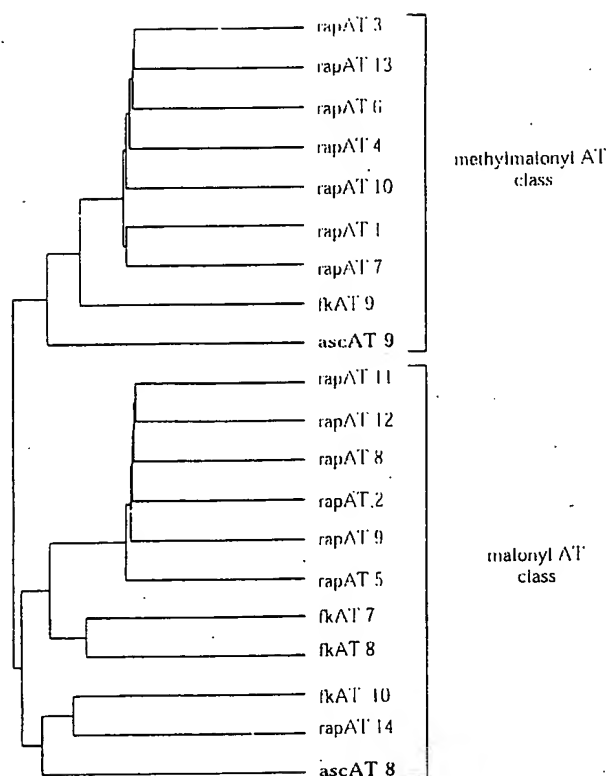


Figure 3. The PILEUP analysis of acyltransferase (AT) domains in these modules. The analysis suggested that the substrates for the identified ATs are acetate (ascAT8) and propionate (ascAT9) respectively.

of rapamycin synthase (RAPS) (7,8) showed high homology to each other, but they have little homology to those for aromatic polyketide synthetic genes (1). The DEBS KS gene probe hybridized to RAPS KS gene or vice versa but neither KS gene probe hybridized to the KS genes of aromatic polyketide synthases (data not shown). We compared the sequences of DEBS1 and RAPS3, and synthesized the PCR primers covering the high homologous sequences around the active sites of the KS genes. DNA fragment (1.1 kb) containing KS active sites was amplified with PCR using the primers. The sequence of the fragment showed high homology to those of DEBS1 and RAPS3 KS genes, especially the active site regions of the KS genes are very well conserved. These results showed that the isolated fragment is an ascomycin KS gene, so we used it as the probe to screen the ascomycin synthase genes.

Fifty-four positive clones were isolated from the cosmid library. Southern-blot analysis of genomic DNA from ascomycin producer cells suggested that the total size of the ascomycin synthase genes is included in 82 kb. We chose the number 44 clone carrying 8 kb insert and determined the sequence completely. Comparison of the deduced amino acid sequence of the clone with the proteins in the database revealed that it contained the consensus active sites of fatty acid synthases and polyketide synthases (12). One complete module containing a KS, an acyltransferase (AT), a dehydratase

(DH), an enoyl reductase (ER) and an acyl carrier protein (ACP), and a part of the module containing a KS and an AT were identified (Fig. 2). The amino acid sequence of these enzyme domains corresponded to the module 12 and 13 of RAPS3 and module 8 and 9 of fkbA.

The sequence of KS domain was conserved well between the macrolide synthases. But other enzymes, AT, DH, ER and ACP showed less homology to each other. The KR domain contains a potential NAD(P)H binding motif, GXGXXAXX-XA (8,12). The KR domains of the modules indicated that the KR is active because it contains LGDSL motif where 4'-phosphopantetheine attaches (12). The PILEUP analysis of AT domains of these modules showed that the substrates are acetate and propionate respectively (Fig. 3). The main structure of ascomycin is speculated to be synthesized with poly-merization of acetate and propionate in the following order; shikimic acid - propionate - propionate - acetate - butyrate - propionate - acetate - acetate - propionate - acetate - pipecolic acid. This sequential arrangement exists only at the C10 to C13 position of ascomycin, which gives a pyranose-ring, in other words tetrahydropyran (Fig. 1). Taken together, we concluded that these modules correspond to modules 8 and 9. DH was identified in the module 8 (Fig. 2), although DH activity in this module is not required for ascomycin biosynthesis. The motif, HxxxGxxxxP is speculated essential for DH activity. The motif of this DH has the mutation of the Gly to Asp (12). DHs are sometimes inactivated by mutation or deletion of amino acids at the active sites, for example the DH of fkbA module 8 and RAPS module 2, 5, 11 and 12 (6-8) contain a five amino acid deletion in the active sites. So the mutated DH in this module is probably an inactive one.

In conclusion, we cloned a part of ascomycin synthetic genes, which code the enzymes for the ascomycin tetra-hydropyranose ring formation.

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